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Effects of photoinitiators on intracellular signaling transduction

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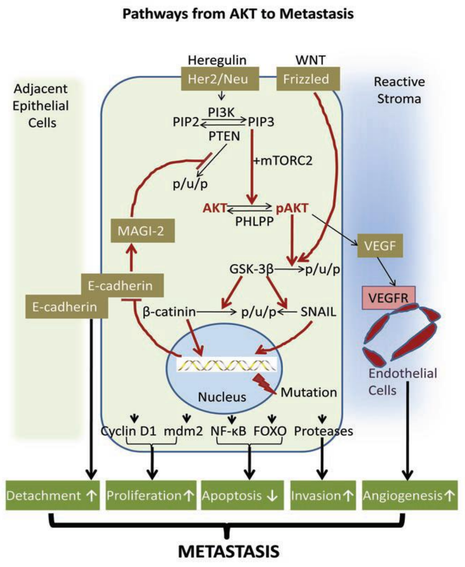
ABSTRACT

Photopolymerization has been widely explored in biomedical and tissue engineering applications due to its widely held potentials in the biomedical field. Photoinitiators embedded within crosslinked scaffold materials could potentially serve as a more effective alternative to present uses of gamma radiation for sterilization of implants and biomaterials. The purpose of this study was to evaluate the cellular toxicity and the intracellular response of three ultraviolet (UV) sensitive photoinitiators including eosin Y, 2-hydroxy-1-[4-(hydroxyethoxy)phenyl]-2methyl-1-propanone (Irgacure 2959) and dimethoxyphenyl acetophenone (DMPA) as well as the subsequent degree of proliferation of free radicals on HN4 cells. WST-1 assay indicated that HN4 cells can tolerate a wide range of concentrations of eosin Y and low concentrations of Irgacure 2959 (≤ 1 mg/ml) but not DMPA. Consistent with these results, eosin Y displayed no effect on intracellular AKT inactivation, but both Irgacure 2959 and DMPA concentration-dependently induced intracellular AKT inactivation. To initiate free radicals, these photoinitiators were exposed to UV light at 365 nm with an intensity of 100 watts for 30 minutes. In all three photoinitiating systems, HN4 cells failed to maintain cell viability and intracellular AKT activity. To further demonstrate the stability of free radicals on cytocompatibility, cell culture medium was mixed with eosin Y to reach a final concentration of 25μl/ml, and this mixture was exposed to UV light for 30 min. The mixture was kept in the dark prior to exposure to the cells. The results illustrate that the free radicals can be stable up to 48 hours, and HN4 cells failed to maintain cell viability and intracellular AKT activity in the eosin Y photoinitiating system after UV exposure. In summary, these results suggest that eosin Y is able to stabilize intracellular AKT activity and cell viability in a wider range than Irgacure 2959 and DMPA. However, the free radicals introduced by UV light significantly inhibit intracellular AKT activation and induce cytotoxicity. Future studies will aim to overcome the cruciality of removing from or significantly limiting free radicals within the photopolymer before biomedical applications.



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METHODS

Photoinitiator preparation
- DMPA and Irgacure 2959 stock solutions: 0, 25, 50, 100, 250 and 500 mg DMPA and Irgacure 2959 separately dissolved in 1 ml of ethanol
- Eosin Y stock solution: 0.1% eosin Y, 4% NVP, and 40% TEOA in PBS
- All resulting photoinitiator solutions protected from light and stored at room temperature until use

Cell culture
HN4 cells derived from a primary SCC of the head and neck cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with high glucose, $_L$-glutamine, 10% (v/v) fetal bovine serum,100 units/ml of penicillin and 100 μg/ml of streptomycin at 37°C in a humid environment with 5% CO_2 prior to any biocompatibility studies

Comparison of photoinitiators
- Cell seeding at density of 50,000 cells/well in 3 ml of cell culture media,
- At 80-90% cell confluence, 40 μl of each DMPA or Irgacure 2959 stock solution added to the culture media to reach a final concentration of 0, 0.5, 1, 2, 5 and 10 mg/ml of each photoinitiator.
- 0, 5, 10, 20 and 40 μl Eosin Y stock solution added to the culture media to reach a final concentration of 0, 2.5, 5, 10 and 20 μl/ml of Eosin Y
- Incubation at 37°C for 30 min prior to harvesting by total cell lysate buffer with protease and phosphatase inhibitors

Western blot analysis
- Total cellular protein expression analyzed for p-AKT, AKT1, and β-actin by standard procedures
- Density of immunoblot bands analyzed using Image J software

Cellular proliferation rate
- Cell seeding at density of 10,000 cells/well and cultured for 2 days to allow the cells adhesion.
- Spent media replace daily with fresh media containing different photoinitiator concentrations
- Incubation with 10% (v/v) 0f WST-1 reagent
- Absorbance measured at 450 nm against a background control as a blank and resulting value subtracted by the absorbance at 650 nm (reference wavelength)

Comparison of photoinitiators after UV exposure
- Fresh cell culture media and photoinitiator stock mixtures at concentrations of 0, 0.5, 1, 2 mg/ml of DMPA and Irgacure 2959 as well as 0, 2.5, 5, 10 μl/ml of Eosin Y
- Exposure to UV radiation at 325 nm for 30 min for derivation of free radicals
- Subjection of HN4 cells to the post-UV treated media for 30 min
- Western blot analysis of total cellular p-AKT and AKT1 expression
- WST-1 assay to determine cellular proliferation rate

Cytotoxicity and cellular response of free radicals
- Fresh cell culture media and Eosin Y stock mixtures at final concentration of 20 μl/m
- Exposure to UV radiation at 325 nm for 30 min for derivation of free radicals
- Subjection of HN4 cells to the post-UV treated media for 30 min after dark resting states of 0, 6, 24, 48 hrs
- Western blot analysis of total cellular p-AKT and AKT1 expression
- WST-1 assay to determine cellular proliferation rate

Statistical analysis
Western blot analysis was repeated at least three times. All the data were expressed as means ± standard deviation (SD) and subjected to analysis of variance (ANOVA) followed by Student *t*-test for unpaired samples. A value of $p < 0.05$ was considered as statistically significant.

MATERIALS

1-vinyl-2 pyrrolidinone (NVP), dimethoxyphenyl acetophone (DMPA), Dimethyl sulfoxide (DMSO), eosin Y, ethanol, phosphate buffer solution (PBS), and triethanolamine (TEOA) were purchased from Sigma-Aldrich (St. Louis, MO). Irgacure 2959 was provided by Ciba Corporation (Newport, DE). Cell proliferation reagent WST-1 was purchased from Roche Applied Science (Indianapolis, IN). Phospho-Akt (Ser473) (p-AKT) antibody was purchased from Cell Signaling Technology (Danvers, MA). AKT1 (559028) antibody was purchased from BD Biosciences Pharmingen (Mississauga, ON, Canada). β-actin (ACTBD11B7) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Goat anti-rabbit antibody conjugated to horseradish peroxidase and goat anti-mouse antibody conjugated to horseradish peroxidase were purchased from Bio-Rad (Hercules, CA).

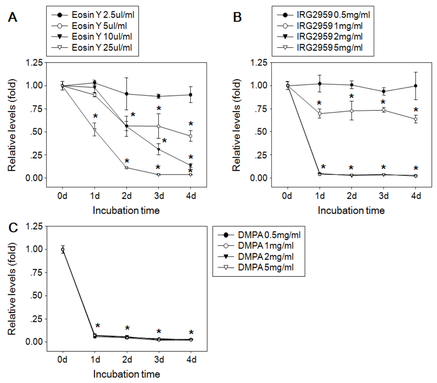

Figure 1: Effects of photoinitiators on cell viability of HN4 cells
HN4 cells were plated at a density of 1×10^4 cells/well into 96-well plates and treated with different concentrations of Eosin Y (**A**), Irgacure 2959 (**B**) or DMPA (**C**) as indicated for 0, 1, 2, 3 and 4 days. The cell viabilities were measured by WST-1 assay at the end of the treatment. The bars are mean ± standard deviation and the symbol * represents statistical significance ($p < 0.05$).

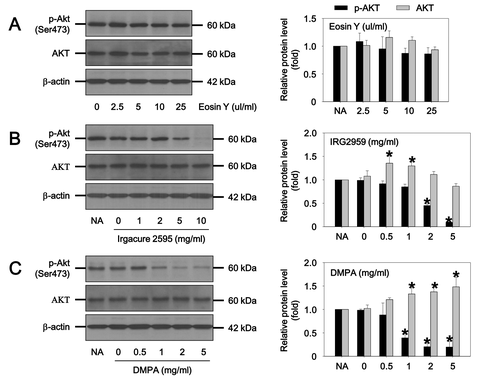

Figure 2: Effects of Eosin Y, Irgacure 2959, and DMPA on AKT activation in HN4 cells
HN4 cells were treated with different concentrations of Eosin Y, Irgacure 2959, and DMPA for 30 min as indicated and the total cell lysates were harvested. The intracellular phospho-AKT (p-AKT) and total AKT levels were analyzed by Western blot analysis. Each positive band was normalized to β-actin and was quantified by NIH ImageJ. The data represents one of three standard examples of these experiments. Each value represents mean ± standard deviation and the symbol * represents statistical significance ($p < 0.05$).

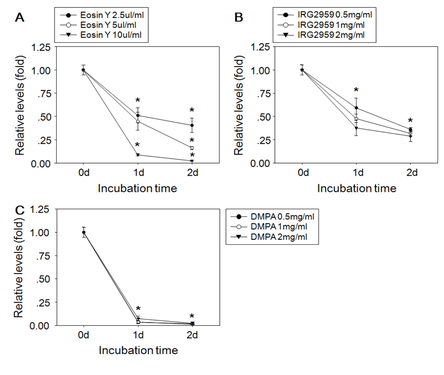

Figure 3: Effects of UV exposed photoinitiators on cell viability of HN4 cells
The cell culture media were first mixed with different concentrations of Eosin Y (**A**), Irgacure 2959 (**B**) or DMPA (**C**) and then the mixtures were exposed to the longwave UV at 365 nm with an intensity of 100 watts for 30 min. After the UV exposure, the mixtures were immediately introduced to HN4 cells for 0, 1 and 2 days of treatment. The cell viabilities were measured by WST-1 assay at the end of the treatment. The bars are mean ± standard deviation and the symbol * represents statistical significance ($p < 0.05$).

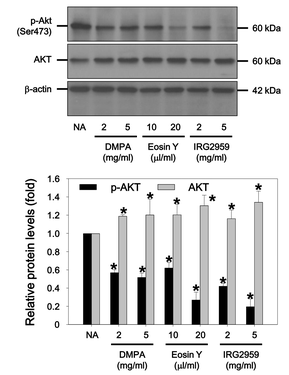

Figure 4: Effects of UV exposed photoinitiators on AKT activation in HN4 cells
The cell culture media were first mixed with different concentrations of Eosin Y, Irgacure 2959 or DMPA and then the mixtures were exposed to the longwave UV at 365 nm with an intensity of 100 watts for 30 min. After the UV exposure, the mixtures were immediately introduced to HN4 cells for 30 min treatment and the total cell lysates were harvested. The intracellular phospho-AKT (p-AKT) and total AKT levels were analyzed by Western blot analysis. Each positive band was normalized to β-actin and was quantified by NIH ImageJ. The data represents one of three standard examples of these experiments. Each value represents mean ± standard deviation and the symbol * represents statistical significance ($p < 0.05$).

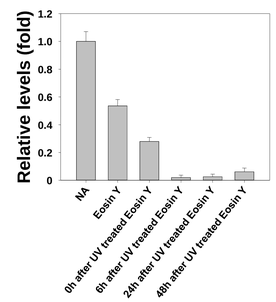

Figure 5: Effects of free radicals introduced by the UV exposure of Eosin Y on cell viability of HN4 cells
The cell culture media were first mixed with Eosin Y to reach a final concentration of 20 μl/ml and the mixtures were exposed to the longwave UV at 365 nm with an intensity of 100 watts for 30 min. After the UV exposure, the mixtures were stored in the dark for 0, 6, 24 and 48 hrs before the cell treatment. HN4 cells were then treated with no addition (NA), 20 μl/ml of Eosin Y without UV exposure or the UV exposed mixtures for 2 days. The cell viabilities were measured by WST-1 assay at the end of the treatment. The bars are mean ± standard deviation and the symbol * represents statistical significance ($p < 0.05$).

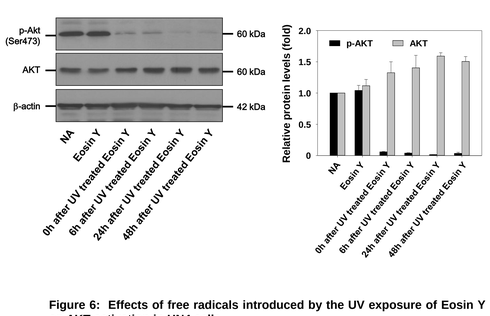

Figure 6: Effects of free radicals introduced by the UV exposure of Eosin Y on AKT activation in HN4 cells
The cell culture media were first mixed with Eosin Y to reach a final concentration of 20 μl/ml and the mixtures were exposed to the longwave UV at 365 nm with an intensity of 100 watts for 30 min. After the UV exposure, the mixtures were stored in the dark for 0, 6, 24 and 48 hrs before the cell treatment. HN4 cells were then treated with no addition (NA), 20 μl/ml of Eosin Y without UV exposure or the UV exposed mixtures for for 30 min and the total cell lysates were harvested. The intracellular phospho-AKT (p-AKT) and total AKT levels were analyzed by Western blot analysis. Each positive band was normalized to β-actin and was quantified by NIH ImageJ. The data represents one of three standard examples of these experiments. Each value represents mean ± standard deviation and the symbol * represents statistical significance ($p < 0.05$).

CONCLUSIONS

Results indicate that among the three photoinitiators tested, Eosin Y proved to posses properties supported cell viability and stable intracellular AKT activity on the most optimal range. However, the stable presence of free radicals induced by photoinitiators causes a failure of HN4 cells to maintain cell viability and intracellular AKT activity. This effect was observed even in the eosin Y photoinitiating system after UV exposure; it is thus vital that free radicals be completely removed and limited before application to biomaterials. Future studies will be specifically directed towards development and synthesis of a targeted anti-cancer drug delivery system incorporating a designated concentration of the photoinitiator for polymerization of cells without detrimental effects of free radicals.

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